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Distribution of Reducible 4-Hydroxynonenal Adduct Immunoreactivity in Alzheimer Disease is Associated with APOE Genotype

KATHLEEN S. MONTINE, ERIN REICH, M. DIANA NEELY, KATHRIN R. SIDELL, SANDRA J. OLSON, WILLIAM R. MARKESBERY, AND THOMAS J. MONTINE

Abstract. Two major risk factors for late-onset familial and sporadic Alzheimer disease (AD), a leading cause of dementia worldwide, are increasing age and inheritance of the ε4 allele of the apolipoprotein E gene (APOE4). Several isoform-specific effects of apoE have been proposed; however, the mechanisms by which apoE isoforms influence the pathogenesis of AD are unknown. Also associated with AD is increased lipid peroxidation in the regions of the brain most damaged by disease. 4-hydroxynonenal (HNE), the most potent neurotoxic product of lipid peroxidation, is thought to be deleterious to cells through reactions with protein nucleophiles. We tested the hypothesis that accumulation of the most common forms of HNE-protein adducts, borohydride-reducible adducts, is associated with AD and examined whether there was a relationship to APOE. Our results demonstrated that reducible HNE adducts were increased in the hippocampus, entorhinal cortex, and temporal cortex of patients with AD. Furthermore, our data showed that the pattern of reducible HNE adduct accumulation was related to APOE genotype: AD patients homozygous for APOE4 had pyramidal neuron cytoplasmic accumulation of reducible HNE adducts, while AD APOE3 homozygotes had both pyramidal neuron and astrocyte accumulation of reducible HNE adducts. This is in contrast to our previous observations that a distinct HNE protein adduct, the pyrrole adduct, accumulates on neurofibrillary tangles in AD patients. We conclude that APOE genotype influences the cellular distribution of increased reducible HNE adduct accumulation in AD.

INTRODUCTION

Alzheimer disease (AD) is a leading cause of dementia worldwide and is the major dementing illness in the United States. Two major risk factors for late-onset familial and sporadic AD, the principal forms of the disease, are increasing age and inheritance of the ε4 allele of the apolipoprotein E gene (APOE4) (1–3). Numerous studies have demonstrated a stratification of risk for AD with the common APOE alleles; APOE4 is associated with the greatest risk for AD, APOE3 is intermediate, and APOE2 is associated with the lowest risk and may be protective (reviewed in 4). Presumably, this genetic susceptibility derives from differences in function among the apolipoprotein E (apoE) isoforms; however, the mechanisms that underlie the isoform-specific effects of apoE on AD pathogenesis remain incompletely understood. Isoform-specific effects of apoE in neuronal culture systems include stabilization of neuronal microtubules, molecular chaperoning of amyloidogenic peptides, antioxidant effects, and neurotrophic actions (5–15).

Polyunsaturated fatty acids are relatively abundant in brain tissue, and lipid peroxidation is a prominent manifestation of oxidative damage to this organ. In AD, the regions of brain most affected by disease also show significantly increased indices of oxidative damage when compared with age-matched controls (16–26). Recently, α-tocopherol, an inhibitor of lipid peroxidation, was shown to slow the clinical progression of AD (27). Deleterious effects of lipid peroxidation include not only direct damage to membranes, but also the liberation of cytotoxins, the most neurotoxic of these being 4-hydroxynonenal (HNE) (28, 29).

Interest in HNE as an effector of oxidative damage to the brain has been fueled by observations that it (a) is a component of amyloid beta peptide–associated oxidative stress in vitro (30), (b) is the most potent neurotoxin among the common products of lipid peroxidation (28, 29), and (c) leads to alterations in neuronal cytoskeleton that mimic some features seen in AD (29). HNE is a potent lipophilic electrophile that will react with a number of cellular nucleophiles (31). It has been shown in liver that the distribution of HNE adducts on macromolecules is 50,000:1 on protein relative to nucleic acids, suggesting that protein modifications are central to the cytopathic effects of HNE (32).

HNE may pursue several different reactions with protein nucleophiles (31–39). The most abundant HNE protein adducts in tissue are Michael adducts that form with several amino acid residues and that exist as cyclic hemiacetals (Fig. 1). HNE also participates in Schiff base chemistry to form imine adducts with protein, although these are much less common than Michael adducts (40). Both the Michael and imine adducts are reducible with borohydride, a reaction that has been exploited in the biochemical and radiochemical detection of protein adducts from products of lipid peroxidation (12, 33–38). In addition to these borohydride-reducible adducts, HNE also reacts with protein amino groups to yield pyrrole

From the Vanderbilt University Medical Center, Department of Pathology, Nashville, Tenn (KSM, ER, DN, KS, SJO, TJM), and the University of Kentucky Medical Center Departments of Pathology, Neurology, and the Sanders-Brown Center on Aging, Lexington, Ky (WRM).

Correspondence to: Thomas J. Montine, MD, PhD, Vanderbilt University Medical Center, Department of Pathology, C3321A Medical Center North, Nashville, TN 37232.

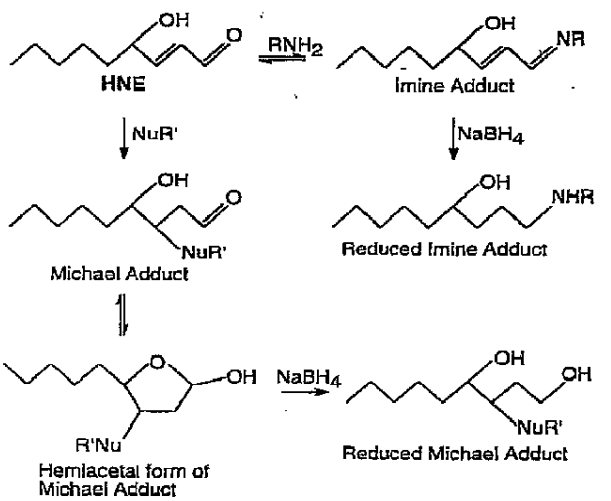

Fig. 1. Diagram of HNE Michael and imine adduct formation. A number of different nucleophiles (NuR') may react with the α, β unsaturated carbonyl moiety of HNE to form Michael adducts that exist in an equilibrium heavily shifted toward the hemiacetal form. Upon reduction with borohydride, the heterocyclic ring structure of the hemiacetal adduct opens to form a stable reduced Michael adduct. Alternatively, HNE may participate in Schiff base-type chemistry to form imine adducts; these also can be reduced by borohydride to form stable adducts.

adducts that are not labile to borohydride reduction (41–43).

We and others have studied HNE pyrrole adducts in AD because of the known neuropathic effects of some pyrroles (44). HNE pyrrole adducts accumulate in hippocampal pyramidal neurons in patients with AD to a much greater extent than in age-matched control patients (41, 45, 46). In two of these studies, accumulation of HNE pyrrole adducts was significantly associated with inheritance of APOE4 (41, 45). This has led us to propose that apoE may influence lipid peroxidation or its effects in an isoform-specific fashion in the brains of patients with AD. The present study has tested this hypothesis further by assaying reducible HNE adduct immunoreactivity in brain tissue from patients with AD and other neurodegenerative diseases, and by investigating potential relationships to APOE genotype.

MATERIALS AND METHODS

HNE, 4-oxononanal (ONA), 2-nonenal (NEA), and 4-hydroxy-2-pentenal (HPE) were obtained as previously described (29, 41). Reagents for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were purchased from BioRad, Hercules, Calif. Anti-ubiquitin rabbit polyclonal antiserum, anti-glial fibrillary acidic protein (GFAP) rabbit polyclonal antiserum, anti-HAM-56 mouse monoclonal antibody, and serum free blocking agent were obtained from Dako, Carpenteria, Calif. 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was purchased from Eastman Kodak Co., Rochester, NY. All other chemicals were obtained from Sigma Chemical Co., St. Louis, Mo.

Antisera Production

Immunogen was produced by reacting 10 mg/ml keyhole limpet hemocyanin (KLH) in 100 mM phosphate buffered saline (PBS) with 1 mM HNE for 4 hours (h) at room temperature, dialyzing away unreacted HNE, reducing the modified KLH with 10 mM sodium borohydride for 30 minutes (min) at room temperature, and then repeated dialyzing. Modification of KLH by HNE was verified by measuring an approximately 15% reduction in protein lysyl amino groups using the TNBS assay (47). The HNE-modified KLH was frozen and shipped to Chemicon International, Temecula, Calif, where it was used to immunize 3 rabbits. Frozen sera from the immunized rabbits was shipped back to our laboratory for analysis.

Immunochemical Assays

Antibody capture assays and immunoblotting were performed as previously described (12, 29). Protein solutions were reduced with 10 mM sodium borohydride for 30 min and then dialyzed before application to the microplate or gel loading well. Protein, either β-lactoglobulin (BLG) or ovalbumin (30 mg/ml in PBS), was added to 10 mM HNE, NEA, ONA, or HPE for 4 h, dialyzed, reduced by 10 mM sodium borohydride for 30 min, and then dialyzed again before being used in competitive antibody capture experiments. The extent of BLG modification by HNE and its congeners was assessed by measuring loss of TNBS reactivity. The dilution of antiserum used in antibody capture assays and immunoblots was 1:1000. Negative control experiments for antibody capture and immunoblotting experiments were preimmune sera at 1:250 dilution reacted with reduced HNE-modified BLG as well as primary antiserum at 1:1000 dilution reacted with HNE-modified BLG that had not been reduced with borohydride.

Immunohistochemistry on formalin-fixed, paraffin-embedded human brain sections was performed as previously described (41, 45). Tissue sections containing hippocampus, entorhinal cortex, and superior and middle temporal cortex were examined for each patient. Prior to application of primary antiserum, tissue sections were reduced with 10 mM sodium borohydride for 30 min and then washed 3 times with PBS. Primary antiserum was used at 1:100 or 1:250 dilution in the immunohistochemistry experiments. In all cases, tissue sections reduced with borohydride were incubated with preimmune serum (1:100 dilution) as the first negative control. The second negative control was primary antiserum (1:250 dilution) preincubated (30 min) with 1 mg/ml borohydride-reduced BLG-HNE before application to reduced tissue sections. Our experimental design permitted a third negative control; consecutive tissue sections from each patient were incubated with primary antiserum exactly as the experimental tissue section but without prior reduction by borohydride. In 3 AD and control cases, cryosections of tissue that had never been fixed were also analyzed by the above method. Double immunohistochemistry was performed using

TABLE
Reducible HNE Adduct Immunoreactivity

	APOE	Total number of patients	Age at death (years ± SEM)	Female: male	Postmortem interval (hours ± SEM)	Immunoreactive neurons (# of patients)	Immunoreactive astrocytes (# of patients)
Alzheimer disease	3/3	7	82.1 ± 3.7	4:3	3.0 ± 0.6	7	6
	4/4	6	72.7 ± 1.5	1:1	2.8 ± 0.5	5	0
Control	3/3	6	77.1 ± 4.9	1:1	3.1 ± 0.8	1	1
	3/4	2	75 and 79	1:1	1.8 and 3.7	0	0
Pick disease	3/3	6	67 ± 2.6	1:1	4.6 ± 0.9	2	2
	3/4	1	78	1:0	3.5	0	0
DLBD-AD	3/4	2	82 and 85	0:2	4.9 and 2.8	2	2
	4/4	2	67 and 75	0:2	2.4 and 5.0	1	0

anti-GFAP antiserum (1:10,000 dilution) or anti-HAM-56 antibody (1:50 dilution) and antiserum 672 (48).

Immunoreactivity involving the entire neuron soma was counted as positive. The extent of neuron immunoreactivity was estimated by determining the percentage of positive neurons in hippocampal sectors CA4 to CA2, CA1, entorhinal cortex, and temporal cortex, and corresponding scores were assigned: 0 for no immunoreactive neurons, 1 for up to 25% positive neurons, 2 for 25 to 75% positive neurons, and 3 for > 75% positive neurons. Astrocyte immunoreactivity was estimated by counting the number of stained granular aggregates adjacent to astrocyte nuclei per 400× field. At least 20 different 400× fields in the hippocampus and entorhinal cortex were evaluated before a section was scored as negative. All cell counting was done by at least two different observers blinded to diagnosis and APOE genotype.

Immunocytochemistry was performed using neuro2A cells grown in tissue culture as described (11). Cultures were exposed to HNE (25 μM) or vehicle (0.0025% ethanol) for 60 min. Cultures were then washed, immediately fixed in 4% paraformaldehyde for 30 min, reduced with borohydride as described above, and then permeabilized with 1% Triton X-100/2% fetal calf serum for 30 min. Following block of nonspecific binding (2 h at room temperature), cells were probed with primary antiserum (1:500 dilution overnight at 4°C), washed, detected with fluorescein-labeled, affinity-purified goat anti-rabbit IgG (1.5 μg/ml for 1.5 h at room temperature), and visualized with a Zeiss Axiovert 135 microscope fitted with LP510+KP560/LP590 excitation/emission filter set.

Patient Selection, Diagnoses, and Statistics

Patients were selected from autopsies performed at Vanderbilt University Medical Center and University of Kentucky Medical Center. AD cases were selected from the most recent autopsies with known homozygosity for APOE3 or APOE4. Control subjects were age-matched individuals without clinical evidence of dementia or other neurological disorders who had annual neuropsychological testing; all had test scores within the normal range. Patients with Pick disease and patients with diffuse Lewy body disease (DLBD) were chosen from the most recent cases. The exclusion criteria were postmortem interval greater than 8 h, stroke, terminal cerebral hypoxia, or any other neurological or systemic disease that could cause dementia. APOE genotype had not been established during life for some non-AD patients; it was determined postmortem on sections of cerebellum according to standard methods (49). The age, gender, and APOE genotype for these groups of patients are presented in the Table.

All brains were fixed in buffered formalin for 7 to 10 days, and then dissected and embedded in paraffin. Complete neuropathological evaluation was performed on each subject to establish a pathological diagnosis or the lack of significant pathological changes in the control group. Standard histopathological criteria were used for diagnosing AD, Pick disease, and DLBD in the appropriate clinical context (50). Comparison of results between patient groups was performed using the chi-squared test. Paired data were compared using t-tests.

RESULTS

Our goal was to raise polyclonal antisera specific to the reducible adducts of HNE with protein. Three different rabbit polyclonal antisera were raised against KLH reacted with HNE and then reduced with borohydride to stabilize the Michael and imine adducts. All three antisera gave similar results in screening antibody capture assays. One antiserum, 672, was chosen for use in all subsequent experiments because it lacked detectable immunoreactivity with nonreduced samples (Fig. 2).

Competitive antibody capture experiments were performed to evaluate the sensitivity and specificity of antiserum 672 for reducible protein adducts of HNE, NEA, HPE, or ONA (Fig. 2). Under the conditions employed, HNE and each of its congeners reduced TNBS-reactive lysyl groups by 10 to 15% relative to unmodified BLG, indicating similar modification of BLG by each compound. Immunoreactivity of antiserum 672 was completely abrogated by increasing concentrations of HNE-modified BLG reduced with borohydride. Reduced NEA-BLG adducts were only weak competitors at the highest concentrations and reduced HPE adducts were noncompetitive, demonstrating that antiserum 672 was specific for reducible HNE-protein adducts as opposed to adducts from other alkenals produced in lower quantities from

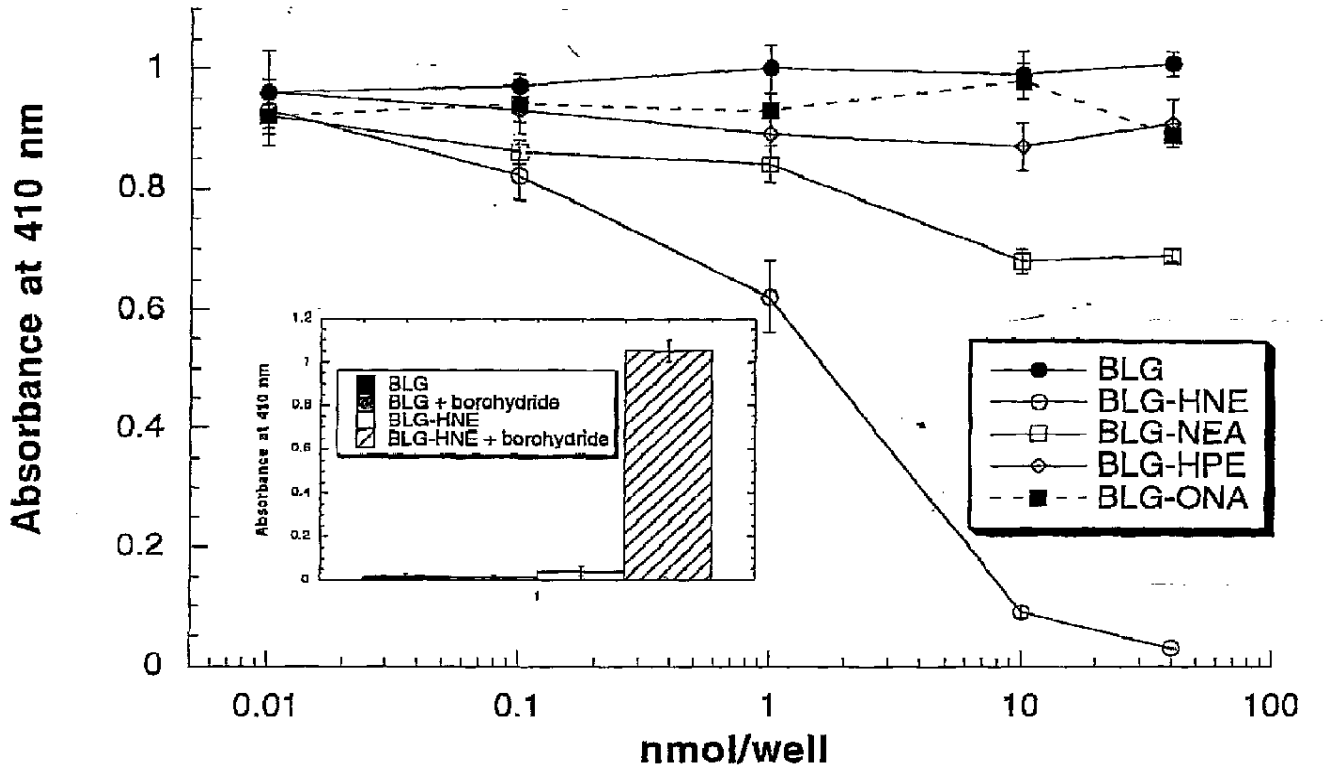

Fig. 2. Antiserum 672 specifically recognizes reduced HNE adducts. Protein, either β-lactoglobulin (BLG) or ovalbumin (30 mg/ml in PBS) was reacted with HNE (10 mM in PBS) or one of its congeners, 2-nonenal (NEA), 4-hydroxy-2-pentenal (HPE), or 4-oxononanal (ONA), for 4 h and then dialyzed. The modified proteins were then reduced with sodium borohydride (10 mM for 30 min) and dialyzed again. Antibody capture assays were performed with antiserum 672 at 1:1000 dilution, goat anti-rabbit alkaline phosphatase conjugated secondary at 1:3000 dilution, and *p*-nitrophenylphosphate as chromagen substrate. Competitive antibody capture experiments used reduced HNE-modified ovalbumin as antigen and reduced modified BLG as competitor. BLG-HNE completely blocked antiserum 672 immunoreactivity. Of the congeners, only NEA was a weak competitor at the highest concentrations. Inset. Antibody capture assays used BLG and modified BLG as antigen. Immunoreactivity was dependent upon HNE adduct formation and borohydride reduction.

lipid peroxidation. ONA is an isomer of HNE that forms pyrrole adducts to the exclusion of Michael and imine adducts (41, 43); ONA-modified protein also was not competitive for antiserum 672. Although not quantitative, similar results were obtained when competing for immunoreactivity on Western blots.

In addition to competitive assays, specificity of antiserum 672 for reducible HNE adducts in neuronal culture was evaluated by immunocytochemical analysis of neuro2A cells exposed to HNE (25 μM for 60 min) or to vehicle (Fig. 3). Neuro2A cells exposed to vehicle lacked any immunoreactivity for antiserum 672, while neurons exposed to HNE showed diffuse immunoreactivity throughout cell body and processes following reduction with borohydride. These results show that in the context of a neuronal cell, immunoreactivity with antiserum 672 requires exposure to HNE.

Antiserum 672 was then used to assay for the presence of borohydride-reducible HNE adducts in tissue sections of hippocampus, entorhinal cortex, and temporal cortex from patients with AD (Table). Age-matched nondemented patients also were examined as controls. In addition, specificity in cerebral degeneration was addressed by examining the same brain regions in patients with Pick disease and DLBD.

Antiserum 672 showed two patterns of immunoreactivity following borohydride reduction of tissue sections, diffuse cytoplasmic staining of some pyramidal neurons and smaller granular aggregates in neuropil and hippocampal white matter (Fig. 4). Perivascular monocytes and vascular smooth muscle cells were immunoreactive with or without borohydride reduction, and therefore were considered to be nonspecific. No other structures were immunoreactive. Specifically, despite demonstration of numerous neuritic plaques and neurofibrillary tangles by ubiquitin immunohistochemistry in AD patients, neither structure was immunoreactive with antiserum 672. Pyramidal neuron cytoplasmic immunoreactivity raises the

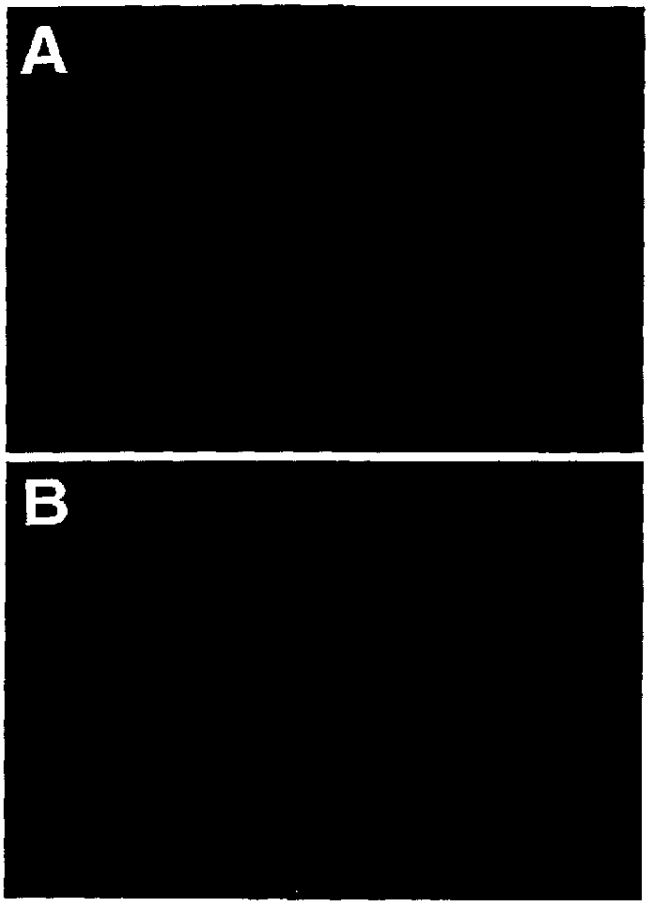

Fig. 3. Cultures of neuro-2A cells exposed to 25 μM HNE (A) or vehicle (0.0025% ethanol) (B) for 60 min, probed with antiserum 672, and then detected with fluorescein-labeled secondary antibody (both photomicrographs are ×630). A. HNE exposed cells show diffuse intense immunoreactivity throughout cell body and processes. B is an image of 2 cells incubated with vehicle only; the procedure was exactly the same as in A except that camera exposure time was twice as long. No immunoreactivity was detected.

possibility that the immunoreactive structure was lipofuscin. However, tissue sections from age-matched control subjects that were replete with lipofuscin granules were not immunoreactive. The intensity and distribution of immunoreactivity was identical in frozen sections of AD and control tissue compared with fixed tissue.

Pyramidal neurons displayed diffuse cytoplasmic immunoreactivity with antiserum 672 following reduction with borohydride in 12 of 13 patients with AD (Fig. 4). All neuron cytoplasmic immunoreactivity was lost when antiserum 672 was preincubated with reduced HNE-modified BLG, when preimmune serum was substituted for antiserum 672, or when antiserum 672 was applied to tissue that had not been reduced with borohydride. The degree of neuron cytoplasmic reactivity with antiserum 672 was a continuum ranging from no immunoreactivity to diffusely staining cytoplasm. We conservatively selected immunoreactivity involving the entire neuron soma as the minimum to be considered a "positive" neuron. The extent of neuron immunoreactivity was estimated in hippocampal sectors CA4-2, CA1, entorhinal cortex, and temporal cortex by determining the percentage of antiserum 672-positive neurons and then scoring as follows: 0 for no immunoreactive neurons, 1 for up to 25% positive neurons, 2 for 25 to 75% positive neurons, and 3 for > 75% positive neurons. Figure 5 presents the average score for antiserum 672-positive pyramidal neurons in these regions. The frequency of antiserum 672-positive neurons was highest in hippocampal sectors, less in the entorhinal cortex, and least in the temporal cortex. AD patients homozygous for APOE4 had a higher frequency score of positive neurons in all regions examined.

Granular immunoreactivity with antiserum 672 that was not localized to neuronal cytoplasm was observed in neuropil for 6 of the 13 AD patients (Figs. 4 and 6). This pattern of immunoreactivity was present only in tissue reduced with borohydride, was completely abrogated by preincubation of antiserum 672 with reduced BLG-HNE, and was absent when preimmune serum was substituted for antiserum 672. These smaller immunoreactive aggregates were either free in neuropil or were associated with astrocyte nuclei, but were never associated with oligodendroglial or microglial nuclei. Moreover, while these immunoreactive structures were more frequent in the hippocampal gray matter, they also were observed in hippocampal white matter, establishing that the immunoreactivity could not belong to a neuron lying just out of plane (Fig. 6). Finally, double immunohistochemistry showed that these immunoreactive structures consistently colocalized with GFAP immunoreactivity, but not with HAM-56 immunoreactivity, a marker of monocytes and microglia (51). This astrocyte immunoreactivity also was present in gray matter of the temporal cortex, albeit less frequently than in the hippocampal gray matter, but was never observed in white matter of the temporal cortex. When present, immunoreactive astrocytes had an average frequency of 4 per 400× hippocampal field (range of 0 to 14 per 400× field), with some immunoreactivity found within the first 2 hippocampal fields examined. At least twenty 400× fields of hippocampus and entorhinal cortex were studied before a tissue section was considered to be nonimmunoreactive.

The 6 AD cases with astrocyte immunoreactivity were all APOE3 homozygotes, including all but one of the AD patients homozygous for APOE3 (Table). None of the 6 AD APOE4 homozygotes had astrocyte immunoreactivity. The association between APOE3 homozygosity and

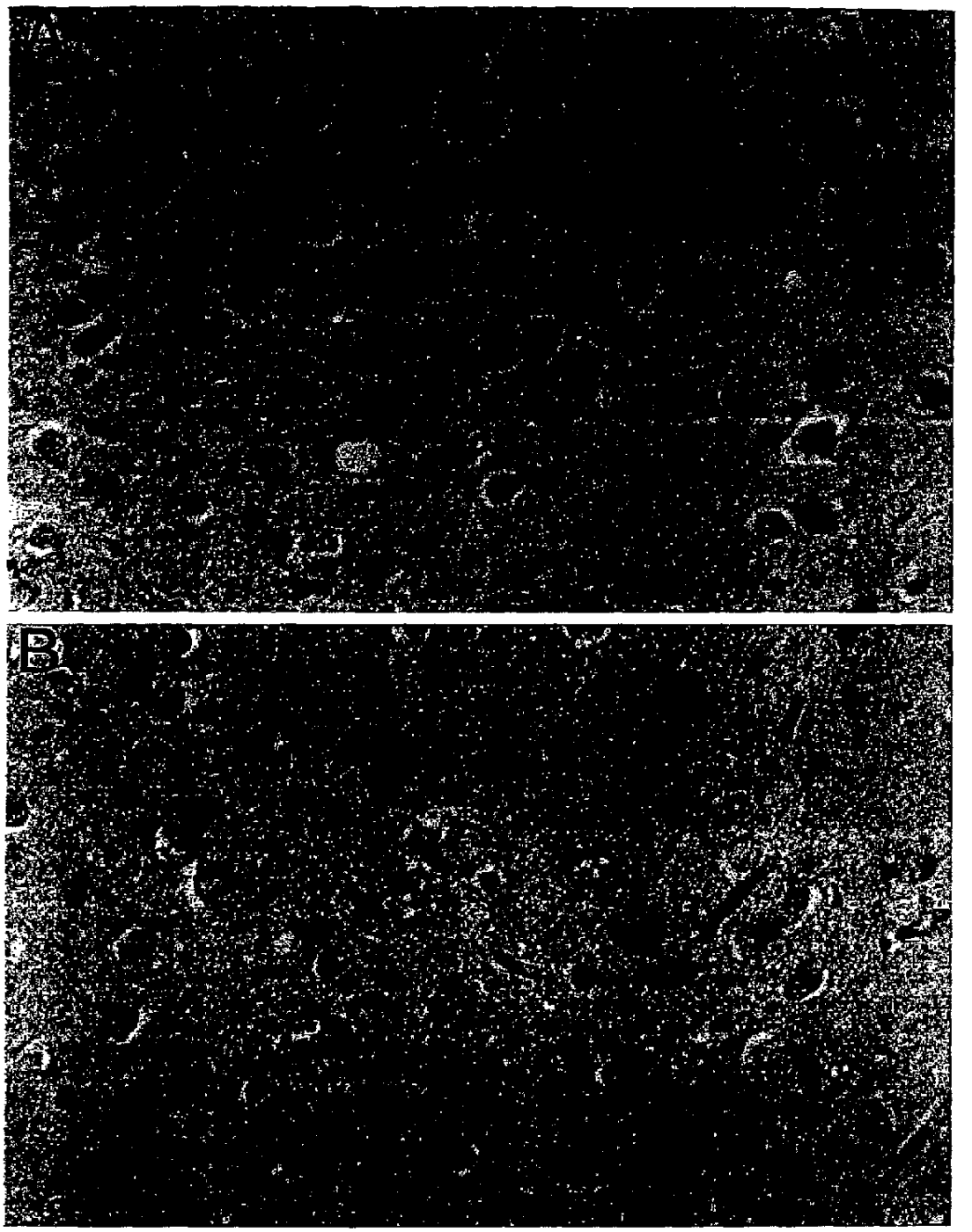

Fig. 4. Photomicrographs of tissue sections reacted with antiserum 672 and preimmune serum. Consecutive 8 μm tissue sections of the same region of entorhinal cortex from an AD APOE3 homozygote were (A) reduced with 10 mM borohydride in PBS for 30 min, washed, and then incubated with antiserum 672 at 1:250 dilution, and (B) incubated with PBS alone for 30 min, washed, and then reacted with antiserum 672 at 1:250 dilution. Both photomicrographs are ×400. Immunoreactivity was dependent upon borohydride reduction and reaction with antiserum 672. The majority of pyramidal neurons show diffuse cytoplasmic immunoreactivity. Smaller aggregates of immunoreactivity also are present in the neuropil and adjacent to astrocyte nuclei (see lower left).

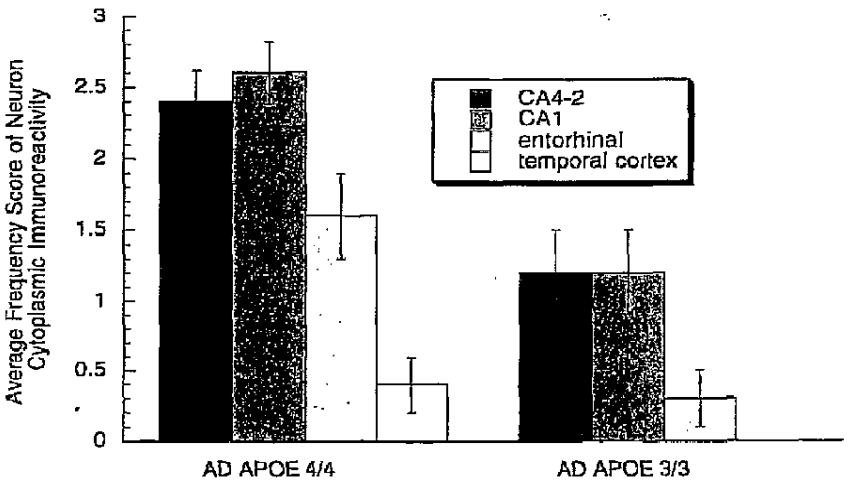

Fig. 5. Regional frequency score for antiserum 672–positive pyramidal neurons in AD patients. The number of 672 positive neurons in hippocampus CA4-2, CA1, entorhinal cortex, and temporal cortex was determined for each AD patient and then scored as either 0 for no positive cells, 1 for < 25% positive neurons, 2 for 25 to 75% positive neurons, or 3 for > 75% neurons. The average regional score (± SEM) was computed for APOE4 (n = 6) and APOE3 (n = 7) homozygotes. Paired analysis for each region's score between APOE 4/4 and APOE 3/3 had $P < 0.05$.

astrocyte immunoreactivity in AD patients was statistically significant (chi-squared test, $P < 0.001$).

Eight age-matched nondemented patients were examined to assess whether antiserum 672 immunoreactivity is also seen in the absence of disease. Only one control patient showed any neuron cytoplasmic immunoreactivity, a value significantly lower than that observed with AD patients (chi-squared, $P < 0.001$). The frequency of antiserum 672-positive neurons in this single control patient was scored as 0, 1, 1, and 0 in hippocampal sectors CA4-2, CA1, entorhinal cortex, and temporal cortex, respectively. The same control patient, an APOE3 homozygote, was the only one to show astrocyte immunoreactivity. Astrocyte immunoreactivity was significantly associated with the presence of AD when analysis was restricted to the 13 AD and control patients homozygous for APOE3 (chi-squared, $P < 0.01$).

The third group, which consisted of 7 patients with Pick disease, was examined to assess specificity among cerebral neurodegenerative diseases. All patients included in this study had involvement of the temporal lobe and had Pick bodies in the hippocampus as demonstrated by ubiquitin immunohistochemistry. Two patients with Pick disease displayed diffuse pyramidal neuron cytoplasmic immunoreactivity with antiserum 672; their average frequency score of positive neurons was 1.7, 2.0, 0.8, and 0.0 for regions CA4-2, CA1, entorhinal cortex, and temporal cortex, respectively. The presence of neuron immunoreactivity for antiserum 672 was significantly higher in AD than in Pick disease when these 20 patients were compared (chi-squared test, $P < 0.01$). There was no difference between the proportion of Pick disease patients and control patients that had reducible HNE adduct immunoreactivity in pyramidal neuron cytoplasm (n = 15).

The 2 Pick disease patients with pyramidal neuron immunoreactivity were the only Pick disease patients with astrocyte immunoreactivity for antiserum 672. The tissue density of astrocyte immunoreactivity was the same as AD patients. The genotypes for these 2 patients were APOE 3/3. The disease specificity of astrocyte immunoreactivity was assessed by comparing all AD and Pick disease patients who had at least one APOE3; this included the 7 AD APOE3 homozygotes and all 7 Pick disease patients. A significantly higher proportion of AD patients displayed astrocyte immunoreactivity than Pick disease patients when controlled for the presence of an APOE3 (chi-squared test, $P < 0.05$); again, there was no difference between patients with Pick disease and controls (n = 15).

Finally, we examined 4 patients with DLBD; however, these cases were complicated by coincident histopathological changes of AD (DLBD-AD). Two of these patients were APOE 3/4 and 2 were APOE4/4. Three of the 4 cases of DLBD-AD had pyramidal neuron cytoplasmic immunoreactivity with antiserum 672 in a pattern identical to the AD cases described above. Both of the DLBD-AD patients with an APOE3 also displayed astrocyte immunoreactivity; neither of the 2 DLBD-AD APOE4 homozygotes had astrocyte immunoreactivity. Although this number of patients is too small to afford valid statistical assessment, the pattern of immunoreactivity in these cases appeared similar to the AD cases described earlier.

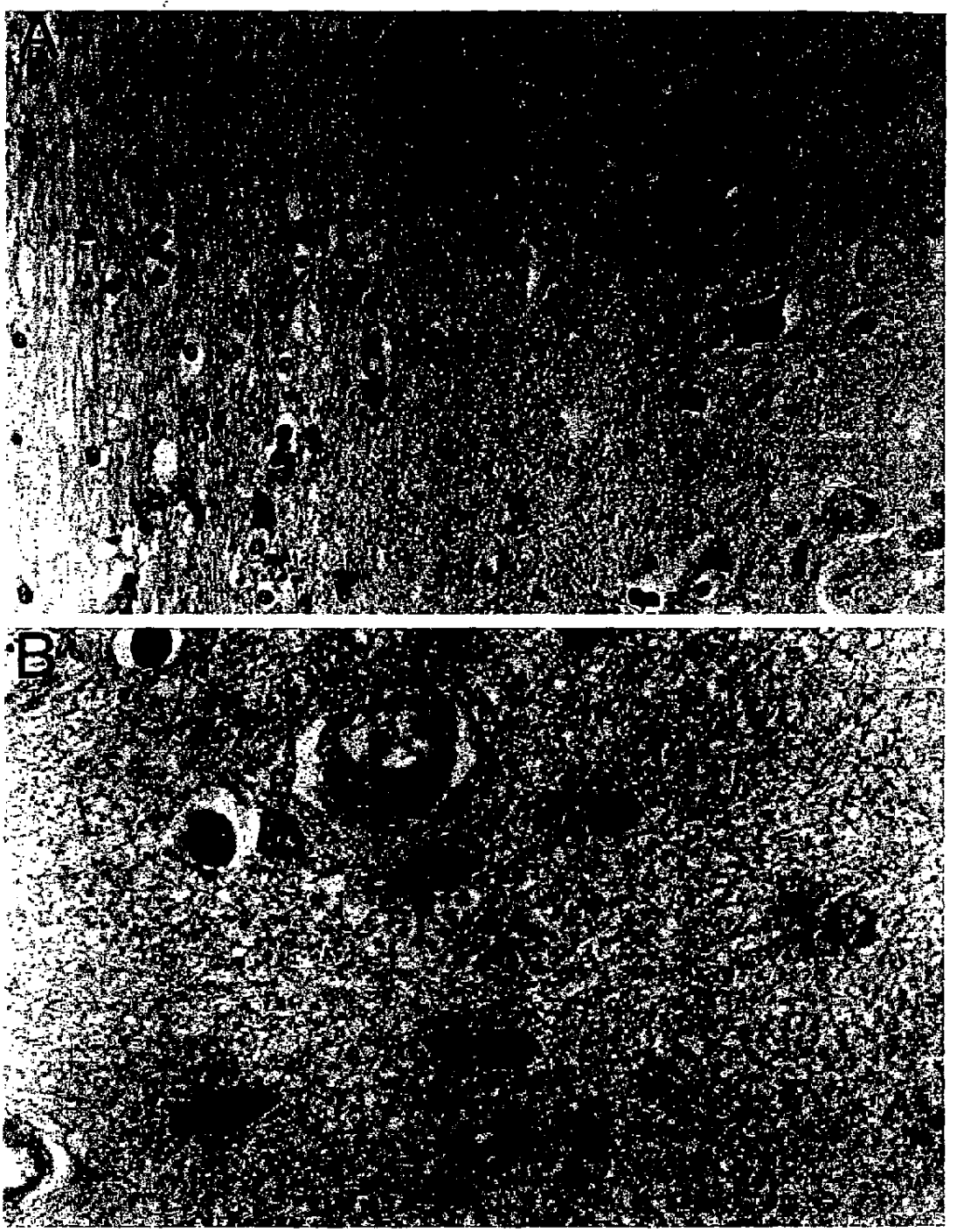

Fig. 6. Antiserum 672 glial immunoreactivity. Tissue sections (8 μm) from 2 different AD APOE3 homozygotes were reduced with 10 mM borohydride for 30 min, washed, and then incubated with antiserum 672. Both photomicrographs are of the hippocampus: (A) shows an immunoreactive pyramidal neuron and granular glial immunoreactivity extending into the fimbrial fibers on the left (×400), (B) shows a pyramidal neuron with surrounding granular immunoreactive aggregates associated with astrocyte nuclei (×1,000).

DISCUSSION

Several studies have implicated a role for lipid peroxidation of brain in the pathogenesis of AD. HNE is an abundant product of lipid peroxidation that has been proposed to contribute to neurodegeneration in AD through modification of cellular proteins; however, this is based largely on experiments using cultured neurons (29, 30, 52). In this study we developed an immunochemical probe for the most abundant chemical forms of HNE-protein adducts, and tested the hypothesis that these reducible HNE adducts accumulate in the relevant brain regions of patients with AD. In addition, we investigated whether there was a relationship between reducible HNE adduct accumulation and APOE genotype. Our results showed that reducible HNE adducts were increased in the hippocampus of patients with AD and that the pattern of HNE adduct accumulation was related to APOE genotype.

Two patterns of reducible HNE adduct immunoreactivity were demonstrated, one in pyramidal neurons and the other in astrocytes. Pyramidal neurons showed diffuse cytoplasmic reactivity with antiserum 672, a pattern similar to what others have observed with in situ assays for increased protein-bound carbonyls, a more general measure of oxidative damage that includes HNE protein adducts (53). The presence of reducible HNE adducts in pyramidal neuron cytoplasm was significantly associated with AD, but not with the patients' APOE genotype, again a result consonant with earlier in situ protein-bound carbonyl studies. Quantification of immunohistochemistry is fraught with technical shortcomings, especially when staining is a continuum as with antiserum 672 in pyramidal neurons. Therefore, we conservatively selected unambiguous immunoreactivity as "positive" and then reported our results as broad ranges of percent neuron positivity, fully acknowledging that these results are semiquantitative. Nevertheless, in all brain regions examined, pyramidal neurons from AD APOE4 homozygotes were more frequently extensively immunoreactive for reducible HNE adducts than AD APOE3 homozygotes.

In addition to immunoreactivity in the cytoplasm of hippocampal neurons, reducible HNE adducts were found in astrocyte cytoplasm, but only in patients with an APOE3, a relationship that held true across all patient groups. Similar to pyramidal neurons, reducible HNE adduct immunoreactivity in astrocytes was significantly associated with AD relative to control patients when analysis was restricted to APOE3 homozygotes. This astrocyte immunoreactivity has not been reported by others using a protein carbonyl assay (53), perhaps a consequence of greater specificity of immunological detection of HNE adducts as opposed to detection of increased protein-bound carbonyls.

How might apoE isoforms influence the tissue distribution of HNE adduct accumulation? ApoE is the major lipoprotein trafficking molecule in the CNS, and others have shown that apoE isoforms differ in their receptor-binding profiles (54, 55). We have shown previously that CNS lipoproteins of AD patients contain changes characteristic of extensive lipid peroxidation, the process that generates HNE (56). We hypothesize that differences in apoE isoform-mediated trafficking of oxidized CNS lipoproteins in AD patients may underlie, at least in part, the differences in tissue distribution of reducible HNE adducts observed in this study.

Previously, we and others have observed immunoreactivity for a different type of HNE adduct, the pyrrole adduct, in pyramidal neurons from the same regions of the brain from AD patients (41, 45, 46). These HNE pyrrole adducts were observed almost exclusively in patients with an APOE4 allele, and in our studies were concentrated on neurofibrillary tangles (41, 45). The biochemical pathways for reducible HNE adduct formation and HNE pyrrole adducts are distinct. In the presence of sulfhydryl groups, HNE pursues reducible adduct formation to the exclusion of pyrrole adduct formation (31, 41). We speculate that pyrrole adduct formation on NFTs in APOE4 homozygotes may be favored by a microenvironment relatively depleted in sulfhydryls compared with APOE3 homozygotes, perhaps in part due to binding of apoE to NFTs and the lack of cysteine in apoE4 that is present in apoE3. In contrast, we envision that HNE-reducible adducts form on cytoplasmic nucleophiles that are present in both APOE3 and APOE4 homozygotes. Also, pyrrole adducts are irreversible in a biological system, while HNE reducible adducts are reversible, at least in part, and this may contribute to differences in their accumulation in tissue. Regardless of the cellular targets, our studies imply that pyramidal neurons in the hippocampus and entorhinal cortex from AD patients with APOE4 appear to be more susceptible to accumulation of both HNE-reducible adducts and pyrrole adducts than disease-matched APOE3 homozygotes.

We selected Pick disease as a form of primary cerebral cortical degeneration that is clearly distinct from AD. Our results showed that HNE adduct accumulation in Pick disease tissue was not significantly different from control patients. The hippocampus and temporal cortex of each Pick disease subject included in this study were involved by disease, thus indicating that the increased HNE adduct accumulation observed in AD patients is not simply an idiosyncrasy of degeneration in these brain regions. It must be stressed that we did not examine the frontal lobe, a region heavily involved by Pick disease, and therefore cannot exclude the possibility of increased oxidative damage at this site (57). Comparison of AD with DLBD is more problematic because some investigators maintain that they represent different morphological expressions of

the same disease processes (58). Our results indicated that, with respect to reducible HNE adduct accumulation in hippocampus and temporal cortex, AD and DLBD-AD are very similar. Overall, our results suggest that accumulation of reducible HNE adducts in hippocampus and temporal lobe is relatively specific to AD among cerebral degenerative diseases; however, others have shown increased immunodetectable HNE adducts in the midbrain of patients with Parkinson disease (59).

In summary, these results show that an increased tissue burden of reducible HNE adducts is a feature of AD, and that the cellular distribution of HNE adduct accumulation is significantly associated with APOE genotype. While our results suggest increased levels of reducible HNE adducts in pyramidal neurons of AD APOE4 homozygotes relative to disease-matched APOE3 homozygotes, our data also show astrocyte accumulation of HNE adducts exclusively in patients with an APOE3. It may be that the overall tissue burden of reducible HNE adducts is similar between AD patients with APOE3/3 and APOE4/4. Indeed, studies using quantitative biochemical indices of lipid peroxidation in tissue homogenates of AD brain, including HNE concentration, have shown significant increases in relevant regions of AD brain compared with control, but have not observed a significant difference between patients with different APOE genotypes (22, 23, 60). We propose that an effect of apoE3 may be to modify the tissue distribution of reducible HNE adducts. If this were so, it could represent a mechanism whereby apoE3 diminishes or delays the neurotoxicity of HNE in brain tissue, potentially contributing to the stratification of risk for AD with different APOE genotypes.

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